Imported Visceral Leishmaniasis: Diagnostic Dilemmas and Comparative Analysis of Three Assays

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The present study evaluates the performances of three noninvasive serological assays for the detection of immunoglobulin G antibodies to leishmania antigen for the diagnosis of imported cases of kala azar (visceral leishmaniasis [VL]) in a country, Kuwait, where the disease is not endemic. A total of 323 individuals including 21 patients with documented cases of VL, 72 individuals with suspected cases of VL, 155 patients with other parasitic infections, and 75 healthy control individuals were tested by indirect hemagglutination assay (IHA; Behring Diagnostics GmbH, Marburg, Germany), indirect fluorescent-antibody assay (IFA; bioMerieux sa, Marcy l’Etoile, France), and a qualitative membrane-based immunoassay with recombinant leishmania antigen K39 (strip-test; Intersep Ltd, Berkshire, United Kingdom). Our data show that IHA is the most sensitive test (100%), followed by IFA (86.6%) and the strip-test (80.0%). The strip-test was the most specific (100%) of the three assays, followed by IFA (93.0%) and IHA (86.0%). However, the strip-test failed to detect at least three confirmed cases of VL. We conclude that IHA is preferred over IFA and the strip-test for the screening of individuals with suspected cases of VL, especially in a country where VL is not endemic and where the number of cases is regular but limited. The details about some of the patients with VL are presented to highlight the diversity of clinical presentations and problems encountered in the diagnosis of VL in a country where VL is not endemic.

Leishmaniasis occurs in five continents and is endemic in tropical and subtropical regions of 88 countries (19). The geographic distribution of leishmaniasis is limited by the distribution of the sandfly, its susceptibility to cold climates, and its capacity to support the internal development of specific Leishmania spp. There are an estimated 12 million cases of leishmaniasis worldwide, 2 million new cases occur each year, and 350 million people are at risk (19).

The disease can present itself in in four different forms in humans, all with devastating consequences: cutaneous, diffuse cutaneous, mucocutaneous, and visceral. The cutaneous forms are the commonest (1.0 million to 1.5 million cases each year), representing 50 to 75% of all new cases (19). Visceral leishmaniasis (VL; 0.5 million cases per year) is the most fatal if it is left untreated, particularly in patients infected with other organisms, such as patients with AIDS (20). Leishmania major and L. tropica generally cause cutaneous leishmaniasis (CL). Exceptional cases have been described, such as visceral outcomes in individuals infected with L. tropica (13). These cases are referred to as visceralotropic leishmaniasis and differ from classical VL in the variable pathologies observed, with several patients not having the typical presentation of VL and low antileishmania antibody titers (8). Coinfection with Leishmania and human immunodeficiency virus (HIV) is emerging as a new and frightful disease and is becoming increasingly frequent. In Europe up to 70% of adult cases of VL are associated with HIV infection, and up to 9% of people with AIDS suffer from newly acquired or reactivated VL (19).

VL is not endemic in Kuwait; however, sporadic cases of cutaneous leishmaniasis have been reported (1, 4, 7). Each year more than 50,000 workers from various developing countries come to work and reside in Kuwait (6). The majority of these workers are from areas where CL (Syria, Iran, Afghanistan) or VL (India, Bangladesh, Nepal) is endemic. In this report we describe imported cases of kala azar to show the diversity of clinical presentation and challenges of diagnosis in a country where the disease is not endemic. In a region where the disease is endemic, kala azar is suspected clinically in the presence of fever, weight loss, and splenomegaly. However, in a country where kala azar is not endemic, the clinical presentation may be different and thus the diagnosis may be delayed. Definitive diagnosis of kala azar still relies on demonstration of Leishmania sp. amastigotes in bone marrow or biopsy material (spleen or liver). The procedures used to retrieve such material are invasive and often are not sensitive. Therefore, a reliable serological test would be of major importance for the screening of patients with suspected VL for further evaluation. The performances of conventional serological assays for the detection of immunoglobulin G (IgG) antibodies to leishmania antigens are being evaluated for their sensitivities, specificities, and cost-effectiveness.

In the study described here we compared the performances of three assays: indirect hemagglutination assay (IHA), indirect fluorescent-antibody assay (IFA), and a qualitative membrane-based immunoassay with a recombinant leishmania antigen, K39 (strip-test; Intersep Ltd., Berkshire, United Kingdom), for the diagnosis of VL in Kuwait.
MATERIALS AND METHODS

Study groups. The study was carried out during the period from 1998 to May 2000 and included the three groups of patients, as described below.

(i) Patients with suspected VL. Seventy-two patients with suspected VL were referred by clinicians for leishmaniology serology. These patients presented with either of the following: prolonged fever for at least 3 weeks with or without hepatosplenomegaly or hematologic cytopenia and weight loss. Clinical suspicion of VL was further supported if the patient was from a Southeast Asian country and/or had traveled to an area of endemicity in the recent past.

(ii) Patients with documented VL. Twenty-one patients with documented VL were evaluated. Diagnosis of VL was confirmed by direct demonstration of Leishmania amastigotes in tissue specimens (liver, spleen) or bone marrow smears. In a few patients with no positive bone marrow smear or a positive biopsy result, the diagnosis was made in the presence of suggestive clinical features (prolonged fever, hepatosplenomegaly, hematologic cytopenia), significant serological titers, and response to specific treatment.

(iii) Control group. The control group included patients with helminth, protozoan, and other parasitic infections frequently seen in Kuwait and healthy controls. This group was included in the study to determine the specificities of IHA, IFA, and the strip-test for the detection of specific antibodies to leishmanial antigen. The group included patients with (i) helminth and protozoan infections (n = 70), (ii) malaria (n = 30), (iii) schistosomiasis (n = 30), (iv) amoebic liver abscess (n = 25), and (v) healthy controls (n = 75 young adults aged 20 to 40 years). The majority of the individuals in the control group were from India, Sri Lanka, and Bangladesh who presented at an outpatient clinic with complaints other than fever, anemia, and hepatosplenomegaly.

Informed consent was obtained from all individuals enrolled in the study. The study was reviewed and approved by the Ethical Committee of the Medical Research Committee of the Faculty of Medicine, Kuwait University, Kuwait.

Screening assays. Individuals with suspected cases of VL and individuals in the control groups were tested by the assays described in the following sections to detect specific IgG antibodies to leishmania antigen.

(i) IHA. The IHA micromethod for leishmaniology serology was performed according to the manufacturer’s instructions (Behring Diagnostics GmbH, Marburg, Germany). Briefly, 5 μl of serum was mixed with 100 μl of IHA reagent (human group O erythrocytes sensitized with soluble, purified L. donovani antigen [Sudan strain]) in V-shaped microtitration wells. The specific antibodies present in the serum sample cross-link the sensitized erythrocytes, and the agglutinated erythrocytes settle down in the well as a carpet formation. A positive IHA result was defined as a titer of ≥1:64 (the manufacturer’s suggested cutoff for L. donovani infection).

(ii) IFA. The serodiagnosis of leishmaniasis by IFA was done with the Leishmania-Spot IF, as described by the manufacturer (bioMerieux sa, Marcy l’Étoile, France). Briefly, serial twofold dilutions of sera were used to cover the spot of the slide coated with L. infantum parasites cultured in vitro. The slide was washed thoroughly after incubation in a moist chamber for 30 min. The slide was then covered with fluorescein isothiocyanate-labeled anti-human immunoglobulin containing Evans blue. After incubation and washing of the slides, the slides were examined under a fluorescence microscope. Titters of 1:80 for adults and 1:40 for children are equivocal, and the samples should be checked by another technique. A titer higher than 1:160 indicates a positive reaction.

(iii) Intersep leishmania strip-test. The Intersep leishmania rapid test strip test; (Intersep Ltd) is a qualitative membrane-based immunosassay that uses a recombinant leishmania antigen for the detection of IgG antibodies to recombinant leishmania antigen K39. K39 has high degrees of sensitivity and specificity for active kala azar. The test was performed as described by the manufacturer. Briefly, 3 drops of serum (~150 μl) are dispensed into the sample well on the test membrane and allowed to soak in. The membrane is precoated with recombinant K39 leishmania antigen on the test line region and anti-protein A antibody on the control line region. The serum sample reacts with the dye conjugate (protein A colloidal gold) which has been precoated onto the membrane. The complex then migrates upward on the membrane to react with recombinant leishmania antigen. The appearance of a red upper band (control) on the strip indicates the presence of IgG and the proper functioning of the test. The appearance of a lower red band indicates the presence of anti-K39 IgG and signifies a positive test result.

A strip-test-positive, IHA-positive (titer, >1:64), and/or IFA-positive (titer, >1:160) patients in the suspected VL group then underwent standard procedures (bone marrow aspiration or tissue biopsy), and coded Giemsa-stained aspirate smears were examined for Leishmania amastigotes. Aspiration was not done for patients in the control groups with known helminth and parasitic infections or for healthy controls. In evaluations of all three assays, the performances were calculated as the ratio of the percentage of true-positive cases to the total number of positive (true and false) cases. Sensitivity was calculated as (TP)/(TP + FN), specificity was calculated as (TN)/(TN + FP), and the positive predictive value (PPV) was calculated as (TP)/(TP + FP), where TP is the number of patients with true-positive results, FN is the number of patients with false-negative results, TN is the number of patients with true-negative results, and FP is the number of patients with false-positive results.

RESULTS

A total of 323 individuals including 21 patients with documented VL, 72 patients with suspected VL, 155 patients with other parasitic infections, and 75 healthy controls were tested by IHA, IFA, and the strip-test for IgG antibodies to leishmania antigen. The performances of the three assays are shown in Tables 1 and 2.

All 21 patients with documented VL had significant levels of IgG antibodies to leishmania antigen by IHA and IFA (titers,
Leishmania parasites in bone marrow smears and/or tissue biopsy specimens. IFA was positive for 17 (23.6%) patients, of which 15 (65.2%) were confirmed to have VL; and the strip-test was positive for 12 (16.7%) patients, all of which 13 (76.5%) patients were confirmed to have VL by direct demonstration of Leishmania amastigotes in their bone marrow smears and were labeled as having confirmed cases of VL.

>1:64 and >1:160, respectively). However, the strip-test was negative for three patients (patients 2, 8, and 12 [Table 1]). As all patients with documented VL were confirmed to have VL by direct demonstration of Leishmania amastigotes in tissue samples or bone marrow smears, these three patients were classified as having false-negative strip-test results. No Leishmania amastigotes were detected in tissue samples from 2 of the 21 patients with VL (patients 5 and 6 [Table 1]); these patients were labeled as having VL on the bases of suggestive clinical features, significant serological titers, and their responses to VL-specific treatment. The antibody titer in patients with VL did not correlate with the disease severity.

The performances of IHA, IFA, and the strip-test for the detection of IgG antibodies to leishmania antigen in patients with suspected VL and in the control group are shown in Tables 2 and 3. IHA was positive for 23 of 72 (31.9%) patients, of which 15 (65.2%) were confirmed to have VL by direct demonstration of amastigotes in their bone marrow aspirates or tissue biopsy specimens. IFA was positive for 17 (23.6%) patients, of which 13 (76.5%) patients were confirmed to have VL; and the strip-test was positive for 12 (16.7%) patients, all 12 of whom (100%) were confirmed to have VL (Table 2). The sensitivity of IHA for the detection of IgG antibodies to leishmania antigen in patients with suspected VL was 100%, the specificity was 86%, and the PPV was 65.2% (Table 3). The performances of IFA and the strip-test are shown in Table 3 (sensitivities, 86.6 versus 80.0%, respectively; specificities, 93.0 versus 100%, respectively). Both IFA and the strip-test failed to detect significant levels of antibodies to leishmania antigen in at least two patients with VL who had positive splenic asperites.

IHA also showed some reactions with sera from patients with helminth infections (n = 3), malaria (n = 2), and amoebic abscesses (n = 2) (Table 2). All IHA-, IFA-, and strip-test-positive patients then underwent standard procedures (bone marrow aspiration or tissue biopsy). Leishmania amastigotes were seen in Giemsa-stained smears of patients with positive IHA (n = 15), IFA (n = 13), and strip-test (n = 12) results (Table 2). No splenic aspiration was done for IHA-positive patients with other parasitic infections. All 75 healthy controls were negative by all three assays.

All 36 patients with VL, including 21 patients with documented VL plus 15 IHA-positive patients with suspected VL who were confirmed to have VL by direct demonstration of Leishmania amastigotes were determined to be from other countries. The majority of these patients were from Southeast Asian countries: 17 were from Bangladesh, 3 were from India, 2 were from Sri Lanka, 5 were from Afghanistan, and the rest were from other countries. The amount of time that the patients spent in Kuwait before they presented with clinical disease, the diversity of their clinical presentations, and serological data for some of the patients with VL are presented in Table 1. All patients with VL were young (ages, 24 to 40 years) adult males. The time that they had spent in Kuwait before they presented clinically at the hospital ranged from 20 days to 23 months. The common presentation of these patients was fever of 3 days’ to 4 months’ duration, anemia, and hepatosplenomegaly. The majority of patients with VL presented with a wide range of typical as well as atypical symptoms that led to a delay in diagnosis. The details for some of these patients are presented in the Appendix to highlight the diversity of clinical presentations and the problems encountered in diagnosis, especially in a country where VL is not endemic and where clinicians are often not familiar with or alerted to leishmania infection.

DISCUSSION

Leishmaniasis gained renewed prominence in the Middle East following the discovery of viscerotropic L. tropica and the emergence of L. donovani as an organism that coinfected patients infected with HIV (4, 5, 7, 8, 11, 12). Thus far, of the four types of clinical presentation of patients with leishmania infection, only CL has been reported in Kuwait (1). No indigenus case of VL has been reported. Here we have reported on 36 cases of VL; however, all VL cases were imported from other countries. Serology and/or direct demonstration of leishmania parasites in bone marrow smears and/or tissue biopsy specimens or a combination of these assays was used to confirm the diagnosis of VL. We also compared the performances of IHA, IFA, and a qualitative membrane-based immunosassay (strip-test) with a 39-kDa recombinant Leishmania sp. antigen to determine the suitability of an assay for the screening of patients with suspected VL for further evaluation and/or for

<table>
<thead>
<tr>
<th>Study group</th>
<th>Total (%) of patients positive</th>
<th>IHA (%)</th>
<th>IFA (%)</th>
<th>Strip-test (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Documented VL patients</td>
<td>21 (100)</td>
<td>21 (100)</td>
<td>19 (90.5)</td>
<td></td>
</tr>
<tr>
<td>Suspected VL patients</td>
<td>72 (31.9)</td>
<td>17 (23.6)</td>
<td>12 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Patients with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helminth and protozoan infections</td>
<td></td>
<td>70 (3)</td>
<td>2 (4)</td>
<td></td>
</tr>
<tr>
<td>Malaria</td>
<td></td>
<td>30 (2)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td></td>
<td>30 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Amoebic liver abscesses</td>
<td></td>
<td>25 (2)</td>
<td>2 (1)</td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
<td>75 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Microscopy of Giemsa-stained splenic aspiration smears was done to detect Leishmania amastigotes. Fifteen of the IHA-positive patients, 13 of the IFA-positive patients, and 12 of the strip-test-positive patients had amastigotes in their bone marrow smears and were labeled as having confirmed cases of VL.

<table>
<thead>
<tr>
<th>Assay</th>
<th>No. of patients with the following result:</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True positive</td>
<td>False positive</td>
<td>False negative</td>
<td></td>
</tr>
<tr>
<td>IHA</td>
<td>15</td>
<td>49</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>IFA</td>
<td>13</td>
<td>53</td>
<td>2</td>
<td>86.6</td>
</tr>
<tr>
<td>Strip-test</td>
<td>12</td>
<td>57</td>
<td>0</td>
<td>80.0</td>
</tr>
</tbody>
</table>

a Sensitivity, specificity, and PPV were calculated as described in Materials and Methods.
specific therapy. The results of the study show that a positive IHA result is a sensitive and reliable indicator of VL. Of the three tests, IHA was rated as the most sensitive (100%) and as a relatively specific test for the detection of antileishmania antibodies. IHA had weakly positive reactions for patients with amebiasis, malaria, and other helminth infections, but the mean log titers for these patients were significantly lower than those for the patients with VL. Such nonspecific cross-reactions have been reported earlier (2, 17, 21). The performance of IFA was comparable to that of IHA. However, IFA is more subjective and requires a special fluorescence microscope and a darkroom facility that is not readily available at most health centers in Kuwait. Furthermore, IHA is more suitable for the handling of large numbers of specimens and costs less than IFA.

The strip-test was the most specific (100%) of the three assays. However, the sensitivity was much lower (80.0%) than those of IHA and IFA. The strip-test failed to detect specific antibodies in three patients with documented VL and two patients with suspected VL who were confirmed to have VL by direct demonstration of *Leishmania* amastigotes in bone marrow smears. The performance of the strip-test with the K39 antigen was comparable to that reported earlier from Sudan (22), but its sensitivity was markedly less than that of a study reported from India (80 to 100%) (18). The reason for this is unclear; but differences in the antibody responses elicited, ethnic backgrounds, the severities of infection, and times since infection may be relevant variables and need to be considered. Furthermore, unlike other forms of anti-leishmania IgG detection, such as by conventional assays like IHA and IFA, which use crude parasite lysates or undefined antigens, the strip-test uses the specific K39 antigen (2). Thus, the highly specific response is explained by the apparent high degree of specificity of anti-K39 antibodies for active VL (2, 16). False-positive K39 responses have been reported earlier (15, 16). However, false-negative results by the strip-test have not been reported earlier. The kinetics of development of anti-K39 responses are still being investigated, but the response does not seem to develop quickly in individuals who spontaneously control the infection (2). The strip-test is a recently introduced rapid test for the detection of VL that detects antibodies to K39 antigen. The performance of this test is still being investigated under different epidemiological conditions (15, 16).

It is well documented that individuals with VL may remain seropositive by conventional serological tests such as IHA for months or even years following successful treatment of the acute infection (2, 17, 21). Therefore, IHA as a sole diagnostic test for patients with clinically suspected VL is not sufficient (21). Clinical symptoms and a history of travel to countries where VL is endemic are extremely useful in establishing a clinical suspicion of VL, and the diagnosis is confirmed by demonstration of parasites in the bone marrow smear.

More than 1 million immigrants from various developing countries reside in Kuwait, and the majority of them come from Southeast Asian countries. According to a recent report from the World Health Organization, more than 90% of the total VL cases seen in the world are reported from India, Bangladesh, Nepal, and Sudan (19). Thus, the immigrants from these countries where VL is endemic are potentially at high risk for the acquisition of leishmania infection while visiting their home countries during holidays. All patients with VL detected in Kuwait had been in the country for a substantial period of time before they presented clinically and were diagnosed with VL. VL is known to have a long incubation period; however, a wide spectrum of clinical presentations of VL in a country where VL is not endemic delayed the times to the suspicion and final diagnosis of VL. It is well documented that introduced or imported diseases pose unique risks to the country into which they are imported. One of these risks relates to the fact that the health service system in countries into which they are imported is ill equipped to deal with nonendemic diseases. Medical and paramedical personnel are often not familiar with or alerted to the potential presence of the disease, which may lead to delays in diagnosis both in the clinic and in the laboratory (6, 9, 10), a fact well illustrated by some of the patients in our study, for whom the diagnosis was made months after the patient first presented to a doctor. A patient’s clinical presentation or coinfection with other relatively more common endemic infections, i.e., amebiasis, malaria, or brucellosis (patients 2, 3, and 4, respectively [see the Appendix]), may also delay a clinical diagnosis and/or investigation for leishmaniasis.

The possibility of autochthonous transmission of VL infection was explored in relation to the presence of an efficient *Phlebotomus* sp. vector in Kuwait (3). However, the chances of autochthonous transmission of VL are remote because of the nonavailability of the infected reservoir and, possibly, because of the low population density of the vector (3). Recently, a zymodeme of *L. major* LON-4 has been isolated from wild animals (*Meriones libycus* and the dog) in a neighboring country, Saudi Arabia (14). Apart from higher risks to affected individuals, there is also a danger of imported diseases being established in a cycle of transmission in the country into which they are introduced. Risks are higher where potential vectors and intermediate hosts of infectious diseases occur and where suitable climatic conditions prevail. Although the transmission of some major tropical diseases such as VL, malaria, and filariasis does not occur in Kuwait, species of potential vectors of these diseases are prevalent in the country, and the risks that these diseases could be established in a cycle of transmission could be high.

In conclusion, we find that IHA has obvious advantages over IFA and the strip-test, especially in a country where VL is not endemic and where the cases of VL are regular but limited. IHA is highly sensitive (100%) and thus can be used as a screening test for all patients with suspected VL before further confirmation by bone marrow aspiration. The drawback is that there is no correlation between a clinical condition and the antibody titer. In view of the changing epidemiological environment, there is a distinct need to be vigilant in the surveillance for kala azar and to maintain a focus on the diagnosis of kala azar.

### APPENDIX

**Patient 1.** Patient 1 (patient 2, Table 1) was a 40-year-old Bangladeshi soldier who presented with a high temperature with sweating, upper abdominal pain, and dysuria for 5 days. He had been in Kuwait for 23 months and was posted close to the Kuwaiti border with Iraq. His blood profile was normal,
and his spleen and liver were not palpable. He was investigated for malaria and urinary tract infection but was negative for both. The serological assays showed a low-positive IHA result (titer, 1:64), a strongly positive IFA result (titer, 1:1,024), but a negative strip-test result. The bone marrow smear was negative; however, a liver biopsy specimen showed *Leishmania* amastigotes. The patient recovered fully with sodium stibogluconate therapy.

**Patient 2.** Patient 2 (patient 5, Table 1) was a 30-year-old Bangladeshi shepherd who was hospitalized with a temperature of 40°C accompanied by chills, nausea, and massive night sweats for 10 days. The patient had recently arrived in Kuwait and was working on a farm. His medical history was uneventful. His blood profile was normal. He was investigated for malaria and brucellosis. He was negative for malaria, but the test for brucella was equivocal. In view of no definitive diagnosis and his close contact with cattle, the patient was put on presumptive therapy for brucellosis. The patient showed no improvement. The patient was then investigated for leishmaniasis. The serological assays showed high levels of antileishmania antibodies by IHA (titer, >4,096) and a positive strip-test result. The bone marrow smear and and liver biopsy were negative for leishmania amastigotes. The patient was given sodium stibogluconate therapy for 21 days and recovered fully.

**Patient 3.** Patient 3 (patient 6, Table 1) was a 30-year-old Indian who presented with fever, fine morbilliform skin rashes, and vomiting for 2 weeks. He had been in Kuwait for about 12 months. He was anemic and had hepatosplenomegaly. His white blood cell count was within the normal range. He was treated for amebiasis on the basis of positive serology for amoebae (titer, 1:128) but showed no improvement. The patient was then screened for leishmaniasis and was found to be positive for leishmania antibodies by all three assays (IHA and IFA titers, 1:512); however, no leishmanial amastigotes were seen on a bone marrow smear or in a liver biopsy specimen. The patient was put on sodium stibogluconate therapy for a trial basis, which was then continued when the patient showed improvement. The patient recovered completely in 8 weeks.

**Patient 4.** Patient 4 (patient 11, Table 1) was a 26-year-old Afghani who presented with fever of 6 weeks’ duration and chronic cough (2 years). Physical examination showed splenomegaly. He had been in Kuwait for 21 months. Laboratory tests showed pancytopenia with a white blood cell count of 2.8 × 10^9/liter that dropped further to 1.6 × 10^9/liter in 5 days. His blood smear was positive for *Plasmodium vivax*, and thus, he received antimalarial treatment. The patient did not respond to antimalarial therapy and continued to have a high fever and night sweats. Serological investigations for leishmaniasis showed high levels of antibodies (titer, >4,096 by IHA) and a positive strip-test result. The infection was confirmed by a positive bone marrow smear. The patient recovered fully after sodium stibogluconate therapy for 8 weeks.

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